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INTRODUCTION

Sample integrity has always been of prime importance to sanitary microbiologists. Over the years, a variety of temperature and storage conditions have been recommended as a means of maintaining sample integrity when immediate coliform analysis was not possible. These recommendations were developed, in large part, from analysis of drinking water samples which usually have a low coliform and nutrient content. However, in river water samples with high (i.e. 100,000/100 ml) initial coliform densities, Henson(1) showed that the population tended to increase, at both refrigeration (2°-6°C) and room temperatures (23°-27°C), when analyzed after 6, 12, and 30 hours of storage. He found the coliform population increases in the room temperature samples to be more than double those observed for the refrigerated samples. Hendricks(2) showed enteric bacteria were capable of growth in waters just slightly above 10°C when provided with nutrients from a nearby sewage outfall, and a similar nutrient effect may be involved in bacterial density changes in samples. Cohen and Shuval(3) observed an order of magnitude increase in total and fecal coliform counts when untreated sewage effluent samples were stored overnight. However, this did not occur if the samples were diluted before storage.

These studies on river water(1) and untreated sewage effluent(3) suggest that samples with high coliform densities display greater population variability during storage than do drinking water samples. However, the same time and temperature conditions are often used for storage of both pristine and polluted water samples. The current recommendation for preserving stream pollution samples is a maximum holding temperature of 10°C and coliform analysis within 8 hours after sampling(4). This study was designed to examine the effects of temperature and length of storage on total coliform densities in unchlorinated primary effluent samples.

METHODOLOGY

Unchlorinated primary effluent sample volumes of 19 liters were obtained from a local treatment plant having a 9.5°-10°C in-plant effluent temperature. Within 90 minutes of sampling, the initial sample processing in the laboratory was completed. In general, processing was as follows: 200 ml sample volumes were placed in 240 ml (8 oz.) containers (Falcon Plastics); sample temperatures in the containers were determined; and containers were placed in 11-liter water baths which had been equilibrated to maintain the desired holding temperature. Measurements of pH were also made at this time and again after 8 hours holding time.

Two methods were used to bring the effluent to the desired holding temperature. In the slow-cool(SC) method, 200 ml volumes were taken from the 19-liter container and placed directly in the appropriate water bath. In the rapid-cool (RC) method, a 2-liter reagent bottle containing about 1800 ml of effluent from the 19-liter container was placed in a -1°C ethylene glycol bath. The effluent was stirred continuously and the temperature monitored during cooling. When the desired temperature was reached, a 200 ml volume of effluent was withdrawn and placed in the appropriate water bath.

The membrane filter technique was used for coliform analysis(4). An aliquot of effluent obtained directly from the 19-liter container was used for zero time SC coliform counts. After cooling the effluent to the appropriate temperature, an aliquot was taken from the 2-liter reagent bottle for zero time RC coliform counts. All sample dilutions were filtered in triplicate through 0.45 micron filters (Millipore, type HAWG 047 S0). The filters were incubated at 35°C for 24 hours on m-Coliform broth(BBL). After incubation, the total coliform colonies were counted and 15 typical sheen colonies were randomly picked from each of the three replicate filters for verification as coliforms(5).

In this study, five temperature conditions were compared: <1°C-SC, <1°C-RC, 5°C-SC, 5°C-RC, and 10°C. There were no experiments done using the 10°C-RC test condition because the initial plant effluent temperatures were always very close to 10°C.

RESULTS

The results of three 8-hour experiments examining the effects of holding time and temperature are presented here. Table 1 shows the temperature and pH measurements for each experiment. In the SC test conditions, the desired sample temperatures were reached within the first 2 hours and remained stable for the rest of the test period. The pH was stable during each of the three experiments except for the 5°C-RC sample in experiment 3, which had an abnormally low initial pH.

The mean membrane filter-total coliform (m-TC) count/100 ml and percent verification for each time interval and test condition are shown in Tables 2, 3, and 4. The mean and standard deviation of the count/100 ml and percent verification for each test condition over the 8-hour holding time are also presented. In experiment 1 (Table 2), <1°C-SC, <1°C-RC, and 10°C holding conditions were examined. The counts showed the <1°C-SC coliform population declining slightly after 6 hours, but over the 8 hours it was relatively stable as was the <1°C-RC population. The verification percentages for the <1°C samples were very similar, with the <1°C-SC sample being slightly lower in mean percent verification than the <1°C-RC sample. During the

8-hour storage period at 10°C, the m-TC counts showed a continual increase, while the percent verification showed a continual decrease.

In experiment 2 (Table 3), an intermediate holding temperature, 5°C-SC was added. There was no significant difference between the <1°C-SC and <1°C-RC counts although the zero time count for <1°C-RC samples was relatively low. The 5°C-SC sample has a low count at 2 hours, but was not significantly different from the <1°C samples for the remainder of the test period. The 10°C sample showed a slight increase after 4 hours. Comparing the mean percent verifications, the <1°C samples were higher than either 5°C-SC or 10°C samples. But in general, the magnitude of the population density differences under the various test conditions in this experiment were not as great as those in experiment 1.

In experiment 3 (Table 4), a 5°C-RC test was added so that <1°C-SC, <1°C-RC, 5°C-SC, 5°C-RC, and 10°C test conditions could be compared. The coliform densities for the <1°C and 5°C samples showed about the same degree of variability. However, for both RC samples, the zero time counts were lower than the SC zero time counts. This may be an effect of rapid-cooling. During the remainder of the test period, the 5°C densities were slightly higher than the <1°C samples. Relatively stable counts were found at <1°C and 5°C as compared to the 10°C sample in

which the population doubled over the 8-hour test period. The stability of the counts at <1°C and at 5°C was confirmed by the percent verification and of the five test conditions, the sample at 10°C had the lowest mean percent verification and the highest standard deviation for verification of any of the samples. In general, the magnitude of the differences in the 10°C counts relative to the other test conditions was greater than in experiment 2, but not as great as in experiment 1.

DISCUSSION AND CONCLUSIONS

This study was designed to examine the effects of various temperatures and storage times on coliform density. Time was not a factor except that the coliform density differences at the various holding temperatures became more accentuated with increasing storage time. There was insufficient testing to determine the relative effect on the coliform population density, of either the rapid-cool or slow-cool treatment. However, the results do suggest that storage temperature is a critical factor in maintaining sample integrity when immediate coliform analysis is not possible. In each of the three experiments, the coliform population in the 10°C samples showed density increases over the 8-hour storage period. These 10°C samples also exhibited the lowest mean percent verification compared to the other holding temperatures. The <1°C samples, while usually having the highest mean percent verification, sometimes showed a density decrease after 6 hours. The samples at 5°C showed little change over the 8-hour test period and the SC samples had a percent verification generally as high as the <1°C samples.

These results suggest that an adequate assessment of the coliform density in the sample source can be provided if the

sample temperature is reduced to 5°C or less with analysis completed in 8 hours. However, these observations may only be applicable to samples containing high coliform densities as analyzed by the membrane filter technique. It should be noted that since the Most-Probable-Number (MPN) confidence limits encompass a wide density range, the differences seen in the membrane filter counts for the three experiments could be masked if the MPN technique were used.

Coliform isolates were verified to determine if false-positive colonies were being counted on the membrane filter. The study showed that there were a higher number of false-positive organisms at 10°C than at the lower temperatures, but there was a great variability, in percentage of the population verified, from one plant effluent sample to the next. This fluctuation suggests that verification is necessary for accurate membrane filter count interpretation, and should include the examination of negative as well as positive colonies on the filter. Very little work has been done on the false-positive and false-negative reactions which occur during membrane filter coliform analysis. One group of investigators has found that false-positive reactions can result from synergistic interactions among microorganisms(6). However, the why of false-negative reactions has not been examined.

The current recommendations for sample preservation(4) do not consider the wide variety of sample sources that can be encountered in pollution surveys. This study has shown that maintenance of bacteriological sample integrity during transport and storage requires consideration of sample temperature during storage, nutrient content and coliform density. Additionally, interpreting results from analyses of stored samples may require further evaluation of the accuracy and reliability of coliform analytical methods. Development of preservation guidelines should include studies examining a wide variety of sample sources and using several analytical techniques. Such studies may result in preservation guidelines which suggest using a variety of storage and analytical methods depending on sample source.

In the meantime, sample integrity remains a problem for microbiologists. But as we continue to learn more about environmental factors affecting coliform populations in samples and to understand more fully the limitations of the analytical techniques, we will be better able to provide accurate assessment of the bacteriological quality of an environment.

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Table 1. Sample temperatures and pH readings recorded during the eight-hour storage period.

Experiment no.	Time (hr)	<1°C-SC		<1°C-RC		5°C-SC		5°C-RC		10°C	
		Temp.	pH	Temp.	pH	Temp.	pH	Temp.	pH	Temp	pH
1	0	10.9	7.2	1.8	7.2	---	---	---	---	12.7	7.2
	2	0.2	---	0.1	---	---	---	---	---	10.2	---
	4	0.2	---	0.2	---	---	---	---	---	9.7	---
	6	0.6	---	0.6	---	---	---	---	---	9.8	---
	8	0.7	7.4	0.8	7.4	---	---	---	---	9.8	7.2
2	0	9.3	7.3	1.6	7.3	9.6	7.3	---	---	10.3	7.3
	2	0.7	---	0.6	---	5.5	---	---	---	10.0	---
	4	0.6	---	0.6	---	5.3	---	---	---	10.0	---
	6	0.5	---	0.5	---	5.2	---	---	---	9.8	---
	8	0.4	7.4	0.4	7.3	5.1	7.3	---	---	9.8	7.2
3	0	10.8	7.3	1.8	7.2	11.4	7.3	7.4	6.0	12.1	7.3
	2	0.7	---	0.7	---	5.6	---	5.6	---	9.9	---
	4	0.5	---	0.5	---	5.4	---	5.4	---	9.7	---
	6	0.5	---	0.5	---	5.2	---	5.4	---	9.6	---
	8	0.5	7.4	0.6	7.2	5.6	7.3	5.6	7.5	9.7	7.3

Table 2. Mean m-TC counts and percent verified for experiment 1.

Time	<1°C-SC		<1°C-RC		10°C	
(hr)	m-TC count/100 ml	Percent verified	m-TC count/100 ml	Percent verified	count/100 ml	Percent verified
0	2.6×10^7	62.3	4.2×10^7	62.3	2.6×10^7	62.3
2	2.5×10^7	75.6	2.0×10^7	78.0	5.0×10^7	53.0
4	2.8×10^7	79.7	2.4×10^7	73.7	6.3×10^7	46.7
6	2.0×10^7	64.3	2.2×10^7	75.7	7.8×10^7	44.3
8	1.6×10^7	71.1	2.9×10^7	75.3	1.4×10^8	26.3
Mean	2.3×10^7	70.6	2.7×10^7	73.0	7.1×10^7	46.5
Standard Deviation	$\pm 0.5 \times 10^7$	±7.4	$\pm 0.9 \times 10^7$	±6.2	$\pm 4.3 \times 10^7$	±13.3

Table 3. Mean m-TC counts and percent verified for experiment 2.

Time (hr)	<1°C-SC		<1°C-RC		5°C-SC		10°C	
	m-TC count/ 100 ml	Percent verified	m-TC count/ 100 ml	Percent verified	m-TC count/ 100 ml	Percent verified	m-TC count/ 100 ml	Percent verified
0	2.3×10^7	75.0	1.4×10^7	80.0	2.3×10^7	75.0	2.3×10^7	75.0
2	1.5×10^7	82.0	1.3×10^7	89.0	1.8×10^7	64.0	1.3×10^7	64.0
4	1.4×10^7	93.0	1.4×10^7	86.0	1.3×10^7	84.0	1.9×10^7	74.0
6	2.3×10^7	87.0	1.2×10^7	78.0	1.5×10^7	69.0	2.8×10^7	67.0
8	9.2×10^6	73.0	1.0×10^7	91.0	1.5×10^7	75.0	3.3×10^7	80.0
Mean	1.6×10^7	82.0	1.3×10^7	84.8	1.7×10^7	73.4	2.3×10^7	72.0
Standard Deviation	$\pm 0.6 \times 10^7$	±8.3	$\pm 0.2 \times 10^7$	±5.6	$\pm 0.4 \times 10^7$	±7.5	$\pm 7.9 \times 10^7$	±6.4

Table 4. Mean m-TC counts and percent verified for experiment 3.

Time (hr)	<1°C-SC		<1°C-RC		5°C-SC		5°C-RC		10°C	
	M-TC count/ 100 ml	Percent verified	m-TC count/ 100 ml	Percent verified	m-TC count/ 100 ml	Percent verified	m-TC count/ 100 ml	Percent verified	m-TC count/ 100 ml	Percent verified
0	1.4×10^7	78.0	8.0×10^6*	80.0*	1.4×10^7	78.0	1.2×10^7	85.0	1.4×10^7	78.0
2	9.5×10^6	89.0	1.2×10^7	89.0	9.9×10^6	80.0	1.3×10^7	71.0	1.4×10^7	60.0
4	1.1×10^7	80.0	1.2×10^7	84.0	1.4×10^7	80.0	1.3×10^7	78.0	2.7×10^7	47.0
6	1.7×10^7	67.0	1.8×10^7	89.0	1.3×10^7	89.0	2.1×10^7	62.0	5.7×10^7	49.0
8	1.2×10^7	84.0	1.1×10^7	80.0	1.7×10^7	76.0	1.7×10^7	69.0	2.8×10^7	73.0
Mean	1.3×10^7	79.6	1.2×10^7	84.4	1.4×10^7	80.6	1.5×10^7	73.0	2.8×10^7	61.4
Std. Dev.	$\pm 0.3 \times 10^7$	±8.2	$\pm 0.4 \times 10^7$	±4.5	$\pm 0.3 \times 10^7$	±5.0	$\pm 0.4 \times 10^7$	±8.8	$\pm 1.8 \times 10^7$	±13.9

* Mean of two instead of three replicates because of laboratory error.